09/381903

420 Rec'd PCT/PTO 27 SEP 1999

Mutants of Gramineae pollen allergens for specific immunotherapy, their preparation and use

The invention relates to modified recombinant allergens (mra) which are derived from allergens which can be obtained from natural raw materials by extraction. Pollen grains from Gramineae, such as Phleum pratense, Lolium perenne, Dactylus glomerata, Poa pratensis, Cynodon dactylon and Holcus lanatus, inter alia, are used as the natural raw material.

Extracts of Gramineae pollen, as employed for diagnostic and therapeutic use, consist of a heterogeneous mixture of proteins and glycoproteins, some of which react with IgE antibodies of allergic patients and are termed allergens by definition. The molecular properties of these allergens enable them to be classified into 6 groups, in association with which the cross-reactivity of the Gramineae species in question is relatively high. The dominant allergen groups (main allergens) are groups 5 and 1, in accordance with the customary allergen classification (Liebers et al., Clin. Exper. Allergy, 26, 494-516 (1996)). The N-terminal amino acid sequences and/or the partial or complete deduced amino acid sequences of groups 5 and 1 of the main allergens are known (Vrtala et al., J. Immunology 151, 4773-4781 (1993) and Bufe et al. FEBS. Lett. 263, 6-12 (1995)). Furthermore, methods for cloning these main allergens have been described (Scheiner et al. Int. Arch Allergy Immunol. 98, 93-96 (1992)).

At present, aqueous extracts of Gramineae pollen are used for the in-vitro diagnosis of type 1 allergies. These extracts are also the basis for in-vitro diagnosis and subsequent specific immunotherapy (Fiebig H., Allergo Journal 7, 377-382 (1995)). The use of native allergen extracts for specific immunotherapy is restricted by the IgE-dependent, allergic reactions

(side reactions) which are induced under these circumstances. For this reason, native allergen extracts can only be administered in doses which are below the side effect threshold. In order to achieve the high allergen concentrations which are required for the therapeutic effect, the extracts are administered by means of several consecutive injections at a concentration which increases up to the maintenance dose. By means of adsorption onto gels, it is possible to use allergen extracts for hyposensitization in a manner which is more efficient and less subject to side effects.

A further improvement was achieved by chemically modifying the allergens to form allergoids, which have a lower reactivity with IgE but which to a large extent retain their immunogenecity (Fiebig H., Allergo Journal 7, 377-382 (1995) and Maasch et al. Clin. Ref. Allergy 5, 89-106 (1987)).

In initial investigations with house dust mite allergens, there are indications that a reduction in the IgE reactivity can be achieved by means of directed amino acid replacement (Smith et al. Mol. Immunol. 33, 339-405 (1996) and Nishiyama et al. Mol. Immunol. 32, 1021-1029 (1995)).

At the moment, the established hyposensitization of patients who are allergic to Gramineae pollen is carried out using natural extracts which comprise all the known allergens and also non-allergenic but immunogenic minor components in substantial concentrations, although, for allergen-specific therapy, only those allergen molecules against which the particular patient is in fact sensitized are required. This means that the allergic patient is unavoidably treated with components which do not contribute to his hyposensitization and which can induce side effects.

As a result of the availability of modified recombinant allergens, individual allergens, or defined mixtures, can be used as pharmaceuticals for the hyposensitization in accordance with the individual sensitization spectrum.

This provides the possibility of a specific, made-to-measure therapy.

The invention was based on the object of discovering novel compounds having valuable properties, in particular compounds which can be used for producing pharmaceuticals.

It has been found that the compounds of the present invention, in the form of the modified recombinant allergens and their salts and solvates, possess very valuable pharmacological properties while at the same time being well tolerated. In particular, they have a hyposensitizing effect.

The compounds can be used as pharmaceutical active compounds in human and veterinary medicine, in particular for therapy in association with allergic diseases and for hyposensitizing allergic patients.

Surprisingly, success has been achieved, within the context of the present invention, in using recombinant allergens, whose amino acid sequences are identical to those of allergen molecules which occur in natural extracts, to construct mutants, by means of genetic manipulation methods which are known per se, which react specifically with T lymphocytes of patients who are allergic to grass pollen, i.e. which stimulate the T lymphocytes to proliferate and synthesize cytokines or which induce anergy in the T lymphocytes, but which exhibit a markedly diminished ability to bind to the IgE antibodies which are present in the serum of the T

lymphocyte donors and to grass pollen allergen-specific IgE from the sera of other patients who are allergic to grass pollen.

This effect, which is not seen either in the case of the naturally occurring allergens or in the case of the recombinant allergens, is desirable because

- the IgE-mediated side effects which otherwise occur during hyposensitization are avoided or are at least strongly diminished,

- it ensures recognition of the modified recombinant allergens by the TH memory lymphocytes of the allergic patients,

- it creates the conditions for normalizing the balance, which is disturbed in the allergic patient, of the variously differentiated TH subpopulations,

- it makes possible a therapeutic effect by means of anergizing and/or eliminating the allergen-reactive T cells and functionally reorienting a specific T cell population which is TH2-dominated to one which is TH0/TH1-aligned,

- the immunoglobulin synthesis can be switched from the formation of spec. IgE antibodies (TH2-controlled), which is typical for the allergic patient, to the preferred synthesis of IgG antibodies (TH1-controlled),

- and, as a result, the condition of the patients can be expected to be markedly improved when they are treated with the novel, modified recombinant allergens.

The invention relates to modified recombinant allergens which are derived from allergens which are obtained

from natural raw materials by extraction. Pollen grains from Gramineae, such as Phleum pratense, Lolium perenne, Dactylus glomerata, Poa pratensis, Cynodon dactylon and Holcus lanatus, inter alia, are used as the natural raw material. In particular, the invention relates to modified recombinant allergens which are derived from the main allergens of groups 1 - 6 and whose reactivity with the IgE antibodies of patients who are allergic to grass pollen is eliminated or at least reduced while that with the T lymphocytes is still retained. The modified recombinant allergens differ from the wild type in that the genes for the allergens have been modified by genetic manipulation methods such that the polypeptides which they encode exhibit substitutions, deletions and/or additions of individual or several amino acids as compared with the wild type. At the same time, the dominant T cell-reactive regions of the modified recombinant allergens (T cell epitopes) are not altered by genetic manipulation.

Preferably, the modified recombinant allergens are derived from the main allergens of group 5 or else of group 1. In particular, the novel allergens are derived from the main Phl p 5b allergen.

Using the single-letter code for amino acids, the sequence of Phl p 5b is as follows:

ADAGYAPATPAAAGAAAGKATTEEQKLIEDINVGFKAAVAAAASVPAADK

1 10 20 30 40 50

FKTFEAAFTSSSKAAAAKAPGLVPKLDAAYSVAYKAAVGATPEAKFDSFV

51 60 70 80 90 100

ASLTEALRVIAGALEVHAVKPVTEEPGMAKIPAGELQIIDKIDAAFKVAA

101 110 120 130 140 150

TAAATAPADDKFTVFEAAFNKAIKESTGGAYDTYKCIPSLEAAVKQAYAA

151 160 170 180 190 200

TVAAAPQVKYAVFEAALTKAITAMSEVQKVSQPATGAATVAAGAATTAAG

201 210 220 230 240 250

AASGAATVAAGGYKV

251 260 265

The invention particularly relates to modified recombinant allergens in which at least one, or a combination, of the regions 16-42, 135-149 and 180-206 of the Phl p 5b polypeptide, consisting of a total of 265 amino acids, is/are not altered. The segments to be preserved are the T cell epitope regions.

The said amino acid residues can also be derivatized. Modifications of the side chains are particularly appropriate in this context.

The amino acid residue abbreviations which are listed above and below stand for the residues of the following amino acids:

Ala = A alanine
Asn = N asparagine
Asp = D aspartic acid
Arg = R arginine
Cys = C cysteine
Gln = Q glutamine
Glu = E glutamic acid
Gly = G glycine

His = H	histidine
Ile = I	isoleucine
Leu = L	leucine
Lys = K	lysine
Met = M	methionine
Phe = F	phenylalanine
Pro = P	proline
Ser = S	serine
Thr = T	threonine
Trp = W	tryptophan
Tyr = Y	tyrosine
Val = V	valine.

In addition, the abbreviations below have the following meanings:

Ac	acetyl
BOC	tert-butoxycarbonyl
CBZ or Z	benzyloxycarbonyl
DCCI	dicyclohexylcarbodiimide
DMF	dimethylformamide
EDCI	N-ethyl-N,N'-(dimethylaminopropyl)carbodiimide
Et	ethyl
FCA	fluoresceincarboxylic acid
FITC	fluorescein isothiocyanate
Fmoc	9-fluorenylmethoxycarbonyl
HOBt	1-hydroxybenzotriazole
Me	methyl
MBHA	4-methylbenzhydrylamine
Mtr	4-methoxy-2,3,6-trimethylphenylsulfonyl
HONSu	N-hydroxysuccinimide
OBut	tert-butyl ester
Oct	octanoyl
OMe	methyl ester
OEt	ethyl ester
POA	phenoxyacetyl
Sal	salicyloyl
TFA	trifluoroacetic acid
Trt	trityl (triphenylmethyl).

Insofar as the abovementioned amino acids are able to occur in several enantiomeric forms, all these forms, and also their mixtures (e.g. the DL forms), are included both in that which is stated above and in that which follows. Furthermore, the amino acids can, for example as constituents of compounds, be provided with appropriate protecting groups which are known per se.

So-called prodrug derivatives, i.e. compounds which are modified with, for example, alkyl or acyl groups, sugars or oligopeptides and which are rapidly cleaved in the organism to form the active novel compounds, are also included in the novel compounds.
These prodrugs also include biodegradable polymer derivatives of the novel compounds as described, for example, in Int. J. Pharm. <u>115</u>, 61-67 (1995).

The novel allergens may possess one or more chiral centres and therefore occur in different stereoisomeric forms. The present invention encompasses all these forms.

Very particular preference is given to modified recombinant allergens which are derived from the following group of polypeptides, which are derived from Phl p 5b:

PM1 ($N^{32} \rightarrow D$, $D^{49} \rightarrow L$, $K^{50} \rightarrow A$)
PM2 ($D^{49} \rightarrow L$, $K^{50} \rightarrow A$)
PM3 ($A^{13} \rightarrow C$)
DM1 (Δ $K^{50} \rightarrow P^{\Delta 132}$, $D^{49} \rightarrow L$)
DM 2 (Δ F^{51} - G^{178}, D^{49} - L, K^{50} - A)
DM2* (Δ F^{51} - G^{178}, 179 - 217 altered sequence)
DM3 (Δ A^{154} - T^{177}, $A^{220} \rightarrow T$)

In the above sequences, the amino acids or amino acid sequences which are modified are indicated in each case.

In this context, PM1 denotes point mutation 1 and has the following sequence (the amino acids which are replaced as compared with Phl p 5b are printed in bold):

ADAGYAPATPAAAGAAAGKATTEEQKLIEDI**D**VGFKAAVAAAASVPAALA

1 10 20 30 40 50

FKTFEAAFTSSSKAAAAKAPGLVPKLDAAYSVAYKAAVGATPEAKFDSFV

51 60 70 80 90 100

ASLTEALRVIAGALEVHAVKPVTEEPGMAKIPAGELQIIDKIDAAFKVAA

101 110 120 130 140 150

TAAATAPADDKFTVFEAAFNKAIKESTGGAYDTYKCIPSLEAAVKQAYAA

151 160 170 180 190 200

TVAAAPQVKYAVFEAALTKAITAMSEVQKVSQPATGAATVAAGAATTAAG

201 210 220 230 240 250

AASGAATVAAGGYKV

251 260 265

The other particularly preferred peptides have the following sequences:

PM2 ($D^{49} \rightarrow L$, $K^{50} \rightarrow A$):

ADAGYAPATPAAAGAAAGKATTEEQKLIEDINVGFKAAVAAAASVPAALA

1 10 20 30 40 50

FKTFEAAFTSSSKAAAAKAPGLVPKLDAAYSVAYKAAVGATPEAKFDSFV

51 60 70 80 90 100

ASLTEALRVIAGALEVHAVKPVTEEPGMAKIPAGELQIIDKIDAAFKVAA

101 110 120 130 140 150

TAAATAPADDKFTVFEAAFNKAIKESTGGAYDTYKCIPSLEAAVKQAYAA

151 160 170 180 190 200

TVAAAPQVKYAVFEAALTKAITAMSEVQKVSQPATGAATVAAGAATTAAG

201 210 220 230 240 250

AASGAATVAAGGYKV

251 260 265

PM3 ($A^{13} \rightarrow C$):

ADAGYAPATPAACGAAAGKATTEEQKLIEDINVGFKAAVAAAASVPAADK
1 10 20 30 40 50

FKTFEAAFTSSSKAAAAKAPGLVPKLDAAYSVAYKAAVGATPEAKFDSFV
51 60 70 80 90 100

ASLTEALRVIAGALEVHAVKPVTEEPGMAKIPAGELQIIDKIDAAFKVAA
101 110 120 130 140 150

TAAATAPADDKFTVFEAAFNKAIKESTGGAYDTYKCIPSLEAAVKQAYAA
151 160 170 180 190 200

TVAAAPQVKYAVFEAALTKAITAMSEVQKVSQPATGAATVAAGAATTAAG
201 210 220 230 240 250

AASGAATVAAGGYKV
251 260 265

DM1 ($\Delta K^{50} \rightarrow P^{\Delta 132}$, $D^{49} \rightarrow L$):

ADAGYAPATPAAAGAAAGKATTEEQKLIEDINVGFKAAVAAAASVPAALA
1 10 20 30 40 50

GELQIIDKIDAAFKVAATAAATAPADDKFTVFEAAFNKAIKESTGGAYDTYK
51 60 70 80 90 100

CIPSLEAAVKQAYAATVAAAPQVKYAVFEAALTKAITAMSEVQKVSQPATG
103 110 120 130 140 150

AATVAAGAATTAAGAASGAATVAAGGYKV
154 160 170 180

DM 2 ($\Delta F^{51} - G^{178}$, $D^{49} - L$, $K^{50} - A$):

ADAGYAPATPAAAGAAAGKATTEEQKLIEDINVGFKAAVAAAASVPAALA
1 10 20 30 40 50

GAYDTYKCIPSLEAAVKQAYAATVAAAPQVKYAVFEAALTKAITAMSEVQK
51 60 70 80 90 100

VSQPATGAATVAAGAATTAAGAASGAATVAAGGYKV
102 110 120 130 137

DM2* (Δ F^{51} - G^{178}, 179 - 217 altered sequence):

Sub A3

This sequence corresponds to that of DM2 where, however, the amino acids of positions 179 - 217 of the starting peptide Phl p 5b additionally exhibit an altered sequence and all the subsequent amino acids are missing.

DM3 (Δ A^{154} - T^{177}, A^{220} $\rightarrow$ T):

ADAGYAPATPAAAGAAAGKATTEEQKLIEDINVGFKAAVAAAASVPAADK

1 10 20 30 40 50

FKTFEAAFTSSSKAAAAKAPGLVPKLDAAYSVAYKAAVGATPEAKFDSFV

51 60 70 80 90 100

ASLTEALRVIAGALEVHAVKPVTEEPGMAKIPAGELQIIDKIDAAFKVAA

101 110 120 130 140 150

TAAGGAYDTYKCIPSLEAAVKQAYAATVAAAPQVKYAVFEAALTKTITAMS

151 160 170 180 190 200

EVQKVSQPATGAATVAAGAATTAAGAASGAATVAAGGYKV

202 210 220 230 240

The invention furthermore relates to a process for preparing modified recombinant allergens by using the polymerase chain reaction and/or its variants. When the peptide sequence is known, the allergens can also be prepared by means of methods of peptide synthesis which are known per se, e.g. the modified Merrifield technique, as described in the literature (e.g. in the standard works such as Houben-Weyl, Methoden der organischen Chemie (Methods of Organic Chemistry), Georg-Thieme-Verlag, Stuttgart;), under reaction conditions which are known and are suitable for the said reactions. In this context, use can also be made of variants which are known per se but which are not mentioned here in detail. It is furthermore possible to liberate the peptides from one of their functional

derivatives by treating the latter with a solvolyzing or hydrogenolyzing agent, and/or convert a basic or acidic peptide into one of its salts or solvates by treating it with an acid or base.

Preferred starting compounds for the solvolysis or hydrogenolysis are those which, in place of one or more free amino and/or hydroxyl groups contain corresponding protected amino and/or hydroxyl groups, preferably those which, in place of an H atom which is connected to an N atom, carry an amino protecting group, e.g. those which, in place of an NH_2 group, contain an NHR′ group (in which R′ is an amino protecting group, e.g. BOC or CBZ).

Starting compounds are also preferred which, in place of the H atom of a hydroxyl group, carry a hydroxyl protecting group, e.g. those which, in place of a hydroxyphenyl group, contain an R″O-phenyl group (in which R″ is a hydroxyl protecting group).

Several - identical or different - protected amino groups and/or hydroxyl groups may also be present in the molecule of the starting compound. If the protecting groups which are present are different from each other, they can in many cases be eliminated selectively.

The expression "amino protecting group" is known generally and refers to groups which are suitable for protecting (blocking) an amino group from chemical reactions but which can be removed readily after the desired chemical reaction has been carried out at other sites of the molecule. Typical groups of this nature are, in particular, unsubstituted or substituted acyl, aryl, aralkoxymethyl or aralkyl groups. Since the amino protecting groups are removed after the desired reaction (or reaction sequence) has taken place, their

nature and size is otherwise not critical; however, those amino protecting groups are preferred which have 1-20, in particular 1-8, C atoms. In connection with the present process, the expression "acyl group" is to be interpreted in the widest possible sense. It encompasses acyl groups which are derived from aliphatic, araliphatic, aromatic or heterocyclic carboxylic acids or sulfonic acids, and, in particular, alkoxycarbonyl, aryloxycarbonyl and, especially, aralkoxycarbonyl groups. Examples of acyl groups of this nature are alkanoyl, such as acetyl, propionyl or butyryl; aralkanoyl, such as phenylacetyl; aroyl, such as benzoyl or toluoyl; aryloxyalkanoyl, such as POA; alkoxycarbonyl, such as methoxycarbonyl, ethoxycarbonyl, 2,2,2-trichloroethoxycarbonyl, BOC or 2-iodoethoxycarbonyl; aralkyloxycarbonyl, such as CBZ ("carbobenzoxy"), 4-methoxybenzyloxycarbonyl or FMOC; arylsulfonyl, such as Mtr. Preferred amino protecting groups are BOC and Mtr, and also CBZ, Fmoc, benzyl and acetyl.

The expression "hydroxyl protecting group" is likewise known generally and refers to groups which are suitable for protecting a hydroxyl group from chemical reactions but which can readily be removed after the desired chemical reaction has been carried out at other sites of the molecule. Typical groups of this nature are the abovementioned unsubstituted or substituted aryl, aralkyl or acyl groups and also alkyl groups. The nature and size of the hydroxyl protecting groups is not critical since they are removed once again after the desired chemical reaction or reaction sequence has taken place; groups having 1-20, in particular 1-10, C atoms are preferred. Examples of hydroxyl protecting groups are, inter alia, benzyl, p-nitrobenzoyl, p-toluenesulfonyl, tert-butyl and acetyl, with benzyl and tert-butyl being particularly preferred. The COOH groups in aspartic acid and glutamic acid are preferably protected in the form of their tert-butyl esters (e.g. Asp(OBut)).

Depending on the protecting group employed, the compounds are liberated from their functional derivatives using, for example, strong acids, expediently using TFA or perchloric acid, but also using other strong inorganic acids, such as hydrochloric acid or sulfuric acid, strong organic carboxylic acids, such as trichloroacetic acid, or sulfonic acids, such as benzenesulfonic acid or p-toluenesulfonic acid. It is possible, but not always necessary, for a supplementary inert solvent to be present. Preferred suitable inert solvents are organic, for example carboxylic, acids, such as acetic acid, ethers, such as tetrahydrofuran or dioxane, amides, such as DMF, halogenated hydrocarbons, such as dichloromethane, and, in addition, also alcohols, such as methanol, ethanol or isopropanol, and water. Mixtures of the abovementioned solvents are also suitable. TFA is preferably used in excess without the addition of another solvent; perchloric acid is used in the form of a mixture consisting of acetic acid and 70% perchloric acid in a ratio of 9:1. The reaction temperatures for the cleavage are expediently between about 0° and 50°; the reaction is preferably carried out between 15 and 30° or room temperature.

The BOC, OBut and Mtr groups can, for example, be preferably eliminated using TFA in dichloromethane or using approximately 3 to 5N HCl in dioxane at 15-30°; the FMOC group can be eliminated using an approximately 5 to 50% solution of dimethylamine, diethylamine or piperidine in DMF at 15-30°.

The trityl group is employed for protecting the amino acids histidine, asparagine, glutamine and cysteine. Depending on the desired end product, it is eliminated using TFA/10% thiophenol, with the trityl group being eliminated from all the amino acids mentioned, or using TFA/anisole or TFA/thioanisole, in which case the

trityl group is only eliminated from His, Asn and Gln and remains on the Cys side chain. Protecting groups which can be removed hydrogenolytically (e.g. CBZ or benzyl) can be eliminated, for example, by treatment with hydrogen in the presence of a catalyst (e.g. a precious metal catalyst such as palladium, expediently on a support such as carbon). Suitable solvents in this context are the abovementioned solvents, in particular, for example, alcohols, such as methanol or ethanol, or amides, such as DMF. As a rule, the hydrogenolysis is carried out at temperatures of between about 0 and 100° and under pressures of between about 1 and 200 bar, preferably at 20-30° and under 1-10 bar. Hydrogenolysis of the CBZ group is, for example, effected satisfactorily on 5 to 10% Pd/C in methanol or using ammonium formate (instead of hydrogen) on Pd/C in methanol/DMF at 20-30°.

An acid can be used to convert a base into the affiliated acid addition salt, for example by reacting equivalent quantities of the base and the acid in an inert solvent, such as ethanol, and then concentrating by evaporation. Acids which yield physiologically harmless salts are particularly suitable for this reaction. Thus, use can be made of inorganic acids, for example sulfuric acid, nitric acid, hydrohalic acids, such as hydrochloric acid or hydrobromic acid, phosphoric acids, such as orthophosphoric acid, or sulfamic acid, and also organic acids, in particular aliphatic, alicyclic, araliphatic, aromatic or heterocyclic monobasic or polybasic carboxylic, sulfonic or sulfuric acids, for example formic acid, acetic acid, propionic acid, pivalic acid, diethylacetic acid, malonic acid, succinic acid, pimelic acid, fumaric acid, maleic acid, lactic acid, tartaric acid, malic acid, citric acid, gluconic acid, ascorbic acid, nicotinic acid, isonicotinic acid, methanesulfonic or ethanesulfonic acid, ethanedisulfonic acid, 2-hydroxyethanesulfonic acid, benzenesulfonic acid, p-toluenesulfonic acid, naphtha-

lenemono- and disulfonic acids and laurylsulfuric acid. Salts with acids which are not physiologically harmless, e.g. picrates, may be used for isolating and/or purifying the compounds of the formula I.

On the other hand, an acid of the formula I can be converted into one of its physiologically harmless metal or ammonium salts by reacting it with a base. In this context, suitable salts are, in particular, the sodium, potassium, magnesium, calcium and ammonium salts, and also substituted ammonium salts, e.g. the dimethyl-, monoethyl-, diethyl- or diisopropyl-ammonium salts, cyclohexyl- or dicyclohexyl-ammonium salts, or dibenzylethylenediammonium salts, and, furthermore, salts with arginine or lysine, for example.

The following steps are necessary for ascertaining the DNA or amino acid sequences:

The allergenic constituents of the extracts, which have been prepared by means of customary methods, are identified and their important physicochemical parameters are characterized. Constituents are identified as being allergens by demonstrating their ability to bind to the IgE antibodies of allergic patients. As a rule, this is done using methods which are known per se, such as SDS-PAGE, isoelectrofocusing and then Western blotting with sera from allergic patients, with only the binding antibodies of the IgE isotype being developed. In this context, care has to be taken to ensure that an adequately large number of types of clinically verified allergic patients (a value of 20 should be set as being the lowest number) are used. Other methods, such as CIE or CRIE, can also be used as alternatives.

These Gramineae pollen allergens which have been identified and characterized in this way can be

prepared analytically such that it is possible to carry out an N-terminal amino acid determination. Furthermore, the allergens can also be purified biochemically and used for preparing monoclonal antibodies. These monoclonal antibodies can, like the IgE antibodies in the sera of allergic patients, be used for the immunological identification and characterization of the allergens from natural sources or of the molecules which are prepared by the recombinant technique.

On the basis of this information on allergens and the means for identifying them, it is possible to clone the allergens using known genetic manipulation methods and to express them as recombinant allergens. The DNA clones of the recombinant allergens which have been isolated and characterized using customary methods are the basis for the modifications which are carried out by means of genetic manipulation and which give rise to the novel, modified recombinantly prepared allergen molecules.

In order to ensure the reactivity of the novel, modified recombinant allergens, it is also necessary to identify the T cell epitopes.

The basis for this is knowledge of the amino acid sequence of the allergen in question or of the corresponding, underlying DNA sequence. As a rule, the amino acid sequence is deduced from the DNA sequence of the recombinant allergens. Consequently, within the context of this invention, the affiliated DNA sequences for every peptide sequence cited are also included, even when these DNA sequences are not explicitly disclosed since they can be derived from the peptide sequences in a known and simple manner.

Based on the amino acid sequence, a series of overlapping oligopeptides is prepared by means of customary

methods, such as solid phase synthesis using modified Merrifield techniques, with the entire sequence of the allergens being covered. Oligopeptides having in each case 6-20, preferably 9-15, amino acid residues may suitably be prepared in this context. Dodecapeptides which are offset by in each case 3 amino acids and which cover the entire sequence of the respective allergen in an overlapping manner are very particularly suitable.

In order to identify the T cell epitopes, T cell clones from patients who are allergic to Gramineae pollen are established by repeated stimulation with the purified, natural or recombinantly prepared allergen in question using the customary method (**Lit.**). For this, it is necessary to establish a representative number of T cell clones which derive from a sufficiently large number of donors.

These T cell clones are incubated with the above-described overlapping peptides and the ability of the latter to stimulate the T cells to proliferate is tested. The proliferation is determined by incorporating $[^3H]$-thymidine by means of methods which are customary per se. Those oligopeptides which induce adequate proliferation of the T cell clones are then regarded as peptide ligands which correspond to the T cell epitopes. The T cell epitopes which have been determined in this manner are used to define T cell-reactive regions of the allergens which, for their part, constitute the basis for constructing the novel modified recombinant allergens.

In order to ensure that modified recombinant allergens react with the T lymphocytes which are found in allergic patients, the primary structures of the T cell-reactive regions which encompass the immunodominant T cell epitopes are partially or completely excluded from alterations.

Genetic manipulation is used to perform mutations in the DNA sequences underlying the remaining regions of the polypeptides (allergens) in order to produce an altered primary structure. This altered primary structure destroys or limits the ability of sequence-dependent continuous B cell epitopes to bind to the IgE antibodies and, due to the formation of a modified tertiary structure as a consequence of the primary modification, completely or partially abolishes the ability of conformation-dependent, possibly discontinuous epitopes to react with their antibodies.

The mutations can be replacements of individual or several amino acids outside the T cell-reactive regions. Such point mutations are introduced into the DNA, which, for example, encodes rPhl p 5b, by means of site-specific mutagenesis using the polymerase chain reaction (PCR). The plasmid pGS13, an expression vector (pMalc) which contains the cDNA for rPhl p 5b, can be used as the template in this context. Gene-specific primers which contain appropriate base replacements and also a new restriction site (Nhe I or Sph I) are used for the PCR. The fragments which are amplified in the PCR and which carry the mutation are ligated one after the other into a cloning vector and the complete product is then recloned into the pMalc expression vector.

Furthermore, mutations can be performed by means of differentially arranged deletions. In order to prepare the deletion mutants, truncated 3'-terminal fragments of the cDNA of rPHl p 5b are prepared in a PCR using gene-specific primers. Relatively large 3'-terminal fragments are removed from the starting vectors (pGS12 or pGS13) by means of restriction at internal cleavage sites and the fragments which were amplified in the PCR, and which are in each case smaller, are ligated in to replace them.

In an analogous manner, mutations involving additions of one or more amino acids can be produced by inserting additional DNA fragments.

The DNA clones which have been mutated by means of genetic manipulation and which encode modified recombinant allergens are recloned into suitable expression vectors and expressed in suitable host organisms. The fusion proteins are purified in a customary manner from the supernatants or disruptions of these host organisms and, after the fusion moiety has been eliminated, the modified recombinant allergens are prepared in the pure state using customary biochemical methods. It is important that the modified recombinant allergens be used for further tests as pure components which correspond to the natural allergens.

The effects of the induced mutations on the allergenicity, i.e. the ability to bind to the IgE antibodies of allergic patients, of the modified recombinant allergens is determined qualitatively and quantitatively by means of the EAST inhibition test. This assay shows whether a substance to be tested (modified recombinant allergen) is identical to, or different from, the natural allergen and/or the recombinant wild type. The extent of the immunochemical relatedness (cross reactivity) can also be quantified. This EAST inhibition test only takes the reaction with IgE antibodies into account.

Those modified recombinant allergen variants which exhibit an inhibitory effect, measured as P_{rel} at 50% inhibition, which is decreased at least by a factor of 10^2 as compared with the natural allergen and/or recombinant wild type are selected as being suitable.

The modified recombinant allergen variants which have been selected in this way are checked to see whether their T cell reactivity has in fact been retained. For this, a set of T cell clones which react with epitopes

in the T cell-reactive regions are taken for testing in the first phase.

Only those modified recombinant allergens which stimulate the selected clones to proliferate are taken into consideration.

In the second phase, oligoclonal T cell lines, which have been established by repeated stimulation with the relevant allergens, are employed for the testing. Once again, only those modified recombinant allergens which at least give rise to a stimulation index (SI) of 50% of the SI of the wild type are taken into consideration.

In the third phase, polyclonal short-term T cell cultures from the peripheral blood of allergic patients are employed for testing.

Apart from the binding of the allergen to the spec. IgE, the allergen-induced, IgE-mediated release of histamine by allergic effect or cells is of patho-physiological importance for the allergic reaction (side effect). The reactivity of the effector cells (basophils and mast cells) and the epitope specificity of the IgE antibodies which are bound by way of FcεRI are also of importance in this context. For this reason, the modified recombinant allergen variants are tested for their ability to induce histamine release by the degranulation of IgE-loaded basophils which are isolated from the blood of allergic patients. In this functional test, the modified recombinant allergen variants which have been selected in accordance with the above selection regime have to exhibit a markedly reduced ability to release histamine.

The modified recombinant allergens which meet these requirements ensure reactivity with the majority of the TH cells which have a regulatory effect and, due to their diminished IgE reactivity, possess the requisite

properties for being employed as therapeutic agents for the allergen-specific immunotherapy (hyposensitization) of patients who are allergic to Gramineae pollen.

The invention furthermore relates to pharmaceutical preparations which comprise one or more modified recombinant allergen(s) according to the present invention, and/or one of their physiologically harmless salts or solvates, and also, where appropriate, additional active compounds and/or auxiliary substances, for treating IgE-mediated allergies.

The invention furthermore relates to a process for producing pharmaceutical preparations, with at least one modified recombinant allergen and/or one of its physiologically harmless salts or solvates being brought into a suitable dosage form together with at least one solid, liquid or semiliquid carrier substance or auxiliary substance.

The invention furthermore relates to the use of the modified recombinant allergens and/or their physiologically harmless salts or solvates for producing pharmaceutical preparations, in particular by a non-chemical route. In this context, they can be brought into a suitable dosage form together with at least one solid, liquid and/or semiliquid carrier substance or auxiliary substance and, where appropriate, in combination with one or more additional active compound(s). The pharmaceuticals are used for immunospecific therapy, i.e. for hyposensitization in association with allergies. It is likewise possible to conceive of using the modified recombinant allergens directly for the immunospecific therapy (hyposensitization) of allergies.

These preparations can be used in human or veterinary medicine as pharmaceuticals. Suitable carrier substances are organic or inorganic substances which are

suitable for enteral (e.g. oral), parenteral or topical administration or for administration in the form of an inhalation spray and which do not react with the novel compounds, for example water, vegetable oils, benzyl alcohols, alkylene glycols, polyethylene glycols, glycerol triacetate, gelatin, carbohydrates, such as lactose or starch, magnesium stearate, talc or yellow soft paraffin. Tablets, pills, coated tablets, capsules, powders, granules, syrups, juices or drops are, in particular, employed for oral use, while suppositories are employed for rectal use, solutions, preferably oily or aqueous solutions and, in addition, suspensions, emulsions or implants are employed for parenteral use, and ointments, creams or powders are employed for topical use. The novel compounds can also be lyophilized and the resulting lyophilates can, for example, be used to produce preparations for injection. The cited preparations can be sterilized and/or comprise auxiliary substances, such as lubricants, preservatives, stabilizers and/or wetting agents, emulsifiers, salts for affecting the osmotic pressure, buffering substances, dyes, flavourants and/or several additional active compounds, e.g. one or more vitamins. For administration as an inhalation spray, use can be made of sprays which comprise the active compound either dissolved or suspended in a propellant gas or propellant gas mixture (e.g. CO_2 or fluorochlorohydrocarbons). Expediently, the active compound is used in this context in micronized form, with it being possible for one or more additional, physiologically tolerated solvent(s), e.g. ethanol, to be present. Inhalation solutions can be administered using customary inhalers.

The compounds and their physiologically harmless salts, can be used for hyposensitizing allergic patients in association with controlling allergic diseases, in particular allergies which are provoked by grasses and grass pollen.

In this context, the novel substances can, as a rule, be administered in analogy with other known, commercially available peptides, in particular, however, in analogy with the compounds which are described in US-A-4 472 305, and are preferably administered in doses of between about 0.05 and 500 mg, in particular of between 0.5 and 100 mg, per dosage unit. The daily dose is preferably between about 0.01 and 2 mg/kg of bodyweight. However, the special dose for each patient depends on a very wide variety of factors, for example on the efficacy of the special compound employed, on the age, bodyweight, general state of health and sex of the patient, on the diet, on the time and route of administration, on the rate of excretion, on the medicinal combination and on the severity of the particular disease to which the therapy applies. Parenteral administration is preferred.

In that which has been stated above, and in that which follows, all temperatures are given in °C. In order to isolate the products, water is added, if necessary, and the mixture is adjusted, if necessary, depending on the constitution of the end product, to pH values of between 2 and 10 and extracted with ethyl acetate or dichloromethane; the phases are separated and the organic phase is dried over sodium sulfate and concentrated by evaporation; the residue is then purified by chromatography on silica gel and/or by means of crystallization.

INSE2

Example 1

Identification of the T cell epitopes for determining the T cell-reactive regions of the main grass pollen allergen Phl p5

Patients who had case histories of the typical symptomatology of a grass pollen allergy (rhinitis) and who gave a positive skin test (prick test) were selected

for establishing T cell lines (TCL) and T cell clones (TCC) which react with the main group 5 grass pollen allergen of timothy grass (Phleum pratense) Phl p5. These patients had circulating specific IgE antibodies with a RAST class ≥ 3.

40 ml of heparinized blood were obtained from each patient. Peripheral mononuclear cells (PBMC) were then isolated from this blood sample by means of the customary method using density gradient centrifugation. Analogous cell isolations were carried out at a later stage when it was necessary to obtain irradiated autologous antigen-presenting cells (APC) for characterizing the TCL and TCC further. After the PBMC had been counted, TCL which reacted to group 5 allergens in vitro were established as follows and as has already been described in detail elsewhere (Lit. 1): in each well of 24-well microculture plates, from 1.5 to 2.0×10^6 PBMC in 1 ml of culture medium (UltraCulture) were stimulated for 7 days in the added presence of natural Phl p5 allergens (in each case 10 μg/well) which had been purified by immunoaffinity chromatography. A total of from 8 to 10 of these cultures were set up. The isolation of Phl p5 by means of immunoaffinity chromatography has been described in detail (Lit. 2). At the end of the 7 days of culture, IL-2 (from 10 to 20 IU/well) was added to the cell cultures for a further 5 to 7 days. All the individual cultures were then pooled and the T cell blasts were enriched by means of density gradient centrifugation; the TCL which were obtained were then tested in a specific lymphocyte proliferation test (see Lit. 1 as well). For this, 2×10^4/ml TCL blasts were in each case cultured with 5×10^4/ml irradiated autologous APCs in triplicate samples in 96-well microculture plates. 10 - 20 μg of Phl p5 allergen were added as the specific antigen stimulus. After 56 hours of incubation, ^{3}H-labelled thymidine (1 μCi/well) was pipetted into the microcultures. After a further 16 hours, the

radioactivity which had been incorporated into the proliferating T cell blasts was measured in a beta counter (Matrix 96). The results were calculated, as the arithmetic mean of the multiple samples, in counts per minute (cpm). The criterion for the quality of the TCL was the stimulation index, which was obtained by relating the cpm values with Phl p5 addition to those without Phl p5 addition.

After they had been selected, the TCLs were cloned (see Lit. 1). For this, 0.3 [lacuna] of TCL blasts/well were cultured in a final volume of 0.2 ml in 96-well microculture plates (round-bottomed) in the added presence of irradiated allogenic PBMC (5×10^4/well), PHA (1.5 g/ml) and IL-2 (25 IU/ml). After 12 to 14 days, the cultures were fed with fresh irradiated PBMC, PHA and IL-2. In addition, a medium replacement, with addition of IL-2 (25 IU/ml), was carried out every 4 to 5 days. An approx. 10 day period without adding irradiated allogenic PBMC elapsed before the Phl p5-specific proliferation test was carried out. The selected TCC were then multiplied in 24-well microculture plates by being repeatedly stimulated with PHA, irradiated allogenic PBMC and IL-2 (50 IU/ml).

After a TCL had been cloned (see below), the specificity of the isolated TCCs was determined as has just been described. Stimulation indices of at least 5 were rated as being positive for the TCCs. The determination of T cell epitopes for defining the T cell-reactive regions on group 5 allergens was also carried out using specific proliferation tests, with 1-2 µg of synthesized dodecapeptide/ml being used for this purpose in each case (see below).

A total of 86 overlapping synthetic dodecapeptides, which were prepared on the basis of the known primary structure of the Phl p 5b allergen as determined by Bufe et al. (Lit. 3), were used for determining the T

cell epitopes. These peptides were prepared using a commercial synthesis kit supplied by CHIRON Mimotopes Peptide Systems/Clayton, Australia. The amino acid sequences of these peptides had a degree of overlap of 9 amino acids (Tab. 1). The reaction of TCC to one of the peptides used in the specific proliferation test was assessed as being positive when the calculated stimulation index was at least 5.

TCCs from 18 patients who were allergic to grass pollen were included in the investigations. From these, success was achieved in isolating 54 T cell clones which reacted specifically with the dodecapeptides which were based on the Phl p 5b sequence. Analysis of these TCCs shows that recognition of peptide ligands is clearly concentrated in 3 immunodominant T cell-reactive regions. Of the 54 T cell clones, 46, corresponding to 85%, react with the peptides of the 3 immunodominant T cell-reactive regions A, B and C of Phl p 5b (Tab. 1a). Only 8 T cell clones reacted with 5 different peptide ligands, with 3 peptides in each case being recognized by 2 different clones. The immunodominant T cell-reactive region **A** encompasses a peptide (27mer) which corresponds to positions 181-207 and which has a core region consisting of amino acids 181-195. 28 of the 54 Phl p 5b-reactive TCCs, corresponding to **51%**, only react with this immunodominant region **A**.

9 (17%) and 9 (17%) of the T cell clones react with the T cell-reactive regions **C** (position 16-48; 33mer) and **B** (position 133-150), respectively. This concentration of the TH cells of the investigated panel of allergic patients on the recognition of 3 immunodominant T cell-reactive regions of the main allergen Phl p 5b makes it possible to construct Phl p 5b mutants in which these regions are not affected by the point mutations, deletion mutations or addition mutations. This creates the prerequisite for these allergen mutants to react specifically with the allergen-reactive TH cells which are

present in allergic patients and to exert a therapeutic influence on these cells.

Tab. 1: Dodecapeptides which are based on the Phl p 5b sequence and which are used for determining the T cell-reactive regions

1	ADAGYAPATPAA	44	KIPAGELQIIDK
2	GYAPATPAAAGA	45	AGELQIIDKIDA
3	PATPAAAGAAAG	46	LOIIDKIDAAFK
4	PAAAGAAAGKAT	47	IDKIDAAFKVAA
5	AGAAAGKATTEE	48	IDAAFKVAATAA
6	AAGKATTEEQKL	49	AFKVAATAAATA
7	KATTEEOKLIED	50	VAATAAATAPAD
8	TEEQKLIEDINV	51	TAAATAPADDKF
9	QKLIEDINVGFK	52	ATAPADDKFTVF
10	IEDINVGFKAAV	53	PADDKFTVFEAA
11	INVGFKAAVAAA	54	DKFTVFEAAFNK
12	GFKAAVAAAASV	55	TVFEAAFNKAIK
13	AAVAAAASVPAA	56	EAAFNKAIKEST
14	AAAASVPAADKF	57	FNKAIKESTGGA
15	ASVPAADKFKTF	58	AIKESTGGAYDT
16	PAADKFKTFEAA	59	ESTGGAYDTYKC
17	DKFKTFEAAFTS	60	GGAYDTYKCIPS
18	KTFEAAFTSSSK	61	YDTYKCIPSLEA
19	EAAFTSSSKAAA	62	YKCIPSLEAAVK
20	FTSSSKAAAAKA	63	IPSLEAAVKQAY
21	SSKAAAAKAPGL	64	LEAAVKOAYAAT
22	AAAAKAPGLVPK	65	AVKQYAATYAA
23	AKAPGLVPKLDA	66	QAYAATVAAAPQ
24	PGLVPKLDAAYS	67	AATVAAAPOVKY
25	VPKLDAAYSVAY	68	VAAAPQVKYAVF
26	LDAAYSVAYKAA	69	APQVKYAVFEAA
27	AYSVAYKAAVGA	70	VKYAVFEAALTK
28	VAYKAAVGATPE	71	AVFEAALTKAIT
29	KAAVGATPEAKF	72	EAALTKAITAMS
30	VGATPEAKFDSF	73	LTKAITAMSEVQ
31	TPEAKFDSFVAS	74	AITAMSEVQKVS
32	AKFDSFVASLTE	75	AMSEVQKVSQPA
33	DSFVASLTEALR	76	EVOKVSOPATGA
34	VASLTEALRVIA	77	KVSQPATGAATV
35	LTEALRVIAGAL	78	QPATGAATVAAG
36	ALRVIAGALEVH	79	TGAATVAAGAAT
37	VIAGALEVHAVK	80	ATVAAGAATTAA
38	GALEVHAVKPVT	81	AAGAATTAAGAA
39	EVHAVKPVTEEP	82	AATTAAGAASGA
40	AVKPVTEEPGMA	83	TAAGAASGAATV
41	PVTEEPGMAKIP	84	GAASGAATVAAG
42	EEPGMAKIPAGE	85	SGAATVAAGGYK
43	GMAKIPAGELOI	86	GAATVAAGGYKV

Tab. 1a: Mapping the T cell-reactive regions of the main grass pollen allergen Phl p 5

TCC	Stimulating peptide ligands (12mers)	Immunodominant T cell-reactive region			Minor epitope
		A	B	C	
DW 8	139-150		+		
DW 14	196-207	+			
DW 16	181-192, 184-195	+			
DW 23	181-192	+			
DW 25	181-192, 184-195	+			
DW 28	184-195	+			
CBH 1	211-222, 214-225				+
CBH 10	211-222				+
JR 6a	22-33, 25-36			+	
JR 6b	136-147, 139-150		+		
JR 7a	28-39, 31-42			+	
JR 7b	136-147, 139-150		+		
JR 9	181-192, 184-195	+			
JR 10	19-30			+	
JR 11	49-60				+
JR 13	181-192, 184-195	+			
JR 15	181-192, 184-195	+			
JR 19a	31-42			+	
JR 19b	136-147		+		
JR 24	97-108, 100-111				+
JR 25	181-192, 184-195	+			
JR 27	184-195	+			
KS 1	181-192, 194-195	+			
KS 2	181-192, 194-195	+			
KS 3	181-192, 194,195	+			
KS 4	181-192, 194-195	+			
KS 5	181-192, 194,195	+			
KSE 18	43-54				+
UD 6	112-123				+
GE 4	136-147, 139-150		+		
GE 7	136-147		+		
GE 12	37-48			+	
AS 4	181-192, 184-195	+			
AS 5	181-192, 184-195	+			
UZH 2	136-147, 139-15		+		
UZ 25	97-108				+

CB 1	190-201, 193-204	+			
CB 2	181-192, 184-195	+			
CB 7	25-36			+	
CB 10	181-192, 184-195	+			
CB 14	181-192	+			
MF 11	184-195	+			
AH 19	16-27			+	
AH 26	139-150		+		
JMD 3	133-144		+		
45		A22	9B	7c	7
II 3.2A 12	31-42			+	
II 12.7F11	196-207	+			
II 12.5C10	187-198	+			
II 17.9E5	184-195	+			
II 17.1D8	184-195	+			
II 17.11C2	184-195	+			
II 17.19A1	193-204	+			
II 17.12F5	25-36			+	
II 17.3C10	49-60, 52-63				+
54		28	9	9	8

Literature:

1. Müller WD, Karamfilov T, Fahlbusch B, Vogelsang H, Jäger L: "Analysis of human T cell clones reactive with group V grass pollen allergens". Int. Arch. Allergy Immunol. 1994, 105:391-396.

2. Jung K, Fahlbusch B, Müller WD, Hermann D, Diener C, Jäger L: "Isolation of timothy (Phleum pratense) allergens using affinity chromatography with monoclonal anti-bodies". Allergy Immunol (Leipzig) 1989, 35:287-294.

3. Bufe A, Schramm G, Keown MB, Schlaak M, Becker WM: "Major allergen Phl p 5b in timothy grass is a novel pollen Rnase". FEBS Letters 1995, 263:6-12.

Example 2

Preparation of point mutants PM1, PM2 ($D^{48} \rightarrow L$, $K^{50} \rightarrow A$) and PM3 ($A^{13} \rightarrow C$) of rPhl p 5b

PM2:

Plasmid pGS13 was used as the starting vector. This is the pMalc vector (Biolabs) which contains the cDNA for the wt rPhl p 5b which is cloned between Bam HI and Hind III sites. Fragments 1 (bp: 1 - 153) and 2 (bp: 141 - 1374) of the cDNA for the rPhl p 5b were amplified in a PCR reaction. The following primers (restriction sites are underlined) were used for this reaction:

Fragment 1:

Sub C5

Phl p 5b sense:
5'-ATATGGATCCATCGAGGGAAGGGCCGATGCCGGCTACGCC-3'

MP1 antisense:

5'-GAACGCTAGCGCCGCAGGGACGCTGGC-3'

Fragment 2:

MP1 sense:

5'-GCGCTAGCGTTCAAGACCTTCGAG-3'

Phl p 5b antisense:

5'-ATATAAGCTTTCCTCTGAAGGAAGGCAACCC-3'

As compared with the wt sequence, the two mutagenesis primers MP1 sense and MP1 antisense contain 6 base replacements which additionally give rise to a new restriction cleavage site for the enzyme Nhe I.

The amplified fragment 1 was digested with Bam HI and Nhe I and cloned into vector pUH89 (Jekel et al., Gene: 154, 55-59; 1995). The resulting plasmid, pGS10, was restricted once again with Nhe I/Hind III, and fragment 2 (Nhe I/Hind III) was incorporated into these cleavage sites. This plasmid, pGS11, comprises the complete cDNA encoding rPhl p 5b but containing the desired base replacements. In order to express the point mutant rPhl p 5b PM2, the mutated cDNA was recloned between the Bam HI and Hind III cleavage sites of the expression vector pMalc. The resulting plasmid was designated pGS21.

The point mutant rPhl p 5b PM1 was prepared in analogy with PM2. It contains, as the result of a PCR error, an additional point mutation: $N^{32} \rightarrow D$.
In order to clone this point mutant, the entire cDNA for rPhl p 5b in vector pGS13 was amplified in a PCR using the following primers.

PCysM1:

5'ATATGGATCCATCGAGGGTAGGGCCGATGCCGGCTACGCCCCGGC
CACCCCGGCTGCATGCGGAGCG-3'

Phl p 5b antisense: see above.

As compared with the wt sequence, the mutagenesis primer PCysM1 contains 3 base substitutions which lead to an alanine residue being replaced with a cysteine residue and which at the same time give rise to a new restriction cleavage site for the enzyme Sph I. The PCR product was cloned directly into the pMalc expression vector (Bam HI/Hind III). The resulting vector was designated pCysM1. The success of the mutagenesis was checked in a restriction analysis using Sph I.

Example 3

Preparation of the deletion mutants DM1 (ΔK^{50} - P^{132}, $D^{49} \rightarrow L$), DM2 (ΔF^{51} - G^{178}, $D^{49} \rightarrow L$, $K^{50} \rightarrow A$) and DM3 (ΔA^{154} - T^{177}, $A^{220} \rightarrow T$)

Sub C7

Plasmid pGS21 (see above) was used as the starting vector for cloning the deletion mutant DM1. The bp 399 - 1374 fragment of the cDNA for rPhl p 5b was amplified in a PCR using the following primers:

MP2 sense:
5'-<u>GCTAGC</u>CGGCGAGCTGCAGATCATCG-3'

Phl p 5b antisense: see above.

Vector pGS21 was restricted with Nhe I and Bam HI and separated from the excised fragment. The PCR product, which had also been restricted with Nhe I and Bam HI, was then ligated into the residual vector. The vector which resulted from this, i.e. pDM1, contains the rPhl p 5b cDNA which has a deletion of 252 bp and which encodes the deletion mutant rPhl p 5bDM1. Deletion mutants DM2 and DM3 were prepared in an analogous manner.

Example 4

Use of the EAST inhibition test to demonstrate the diminished allergenicity (IgE reactivity) of the recombinant Phl p 5b mutants

The binding of the allergens by the IgE antibodies is the basic prerequisite for the allergen-specific activation of the effector cells (mast cells, basophils, inter alia) in type I allergy. The allergen-specific inhibition of the enzyme/allergen sorbent test (EAST) is the best means for qualitatively and quantitatively recording the binding of the allergens to IgE antibodies. The EAST inhibition test is carried out as follows. Microtitre plates are coated with allergen (natural or recombinant Phl p 5 or Phl p 5b) (1 μg/ml). After the unbound allergen molecules have been removed by washing, non-specific plastic binding sites are blocked with bovine serum albumin (0.5%). Anti-IgE from allergic patients, as a representative pool of 10-30 donors or as an individual serum, is incubated, in a suitable dilution, with the allergen-coated microtitre plates. The bound allergen-specific IgE antibodies are quantified using enzyme-coupled anti-IgE antibodies (e.g. alk. phosphatase-a-IgE antibodies). This binding is inhibited by soluble allergen or the substance to be tested (allergen mutants) in dependence on the concentration. The inhibition curve obtained with the purified natural allergen Phl p 5b is used as the reference.

The inhibition curves depicted in Fig. 1 are obtained with the representative allergen patient serum pool Bor 18/100 (20 donors).

rPhl p 5b (wild type) and PM3 exhibit binding curves which are similar to that obtained with natural Phl p 5b which has been purified by affinity chromatography. Minor differences are visible due to a better

inhibitory effect in the lower range and to poorer inhibition at high concentrations. While the reason for this is unknown, it might be accounted for by confirmational epitopes which differ to a minor extent.

Point mutant PM1 exhibits this effect in the higher range to a somewhat greater degree. The deletion mutants DM1 and DM3 exhibit a markedly decreased inhibitory effect. This substantiates the strongly reduced allergenicity of these allergen mutants, which, as a consequence, are comparable with chemically modified allergens (allergoids).

Deletion mutants DM2 and DM2* exhibit an extremely low inhibitory effect on the allergen-IgE reaction. This shows that the allergenicity of these mutants has to a large extent been eliminated. While a different serum pool from allergic patients (We 6/97) and also the individual sera from allergic patients II3, II12 and II17 exhibit slight variations in their inhibitory curves with the mutants, they nevertheless confirm that deletion mutants DM1 and DM3 exhibit greatly reduced allergenicity (Figs. 2 - 5). Apart from a low residual activity, the inhibitory effect of deletion mutants DM2 and DM2* is eliminated. Point mutations PM1 and PM3 exhibit either no reduction, or only a reduction which is for the most part slight, in allergenicity (e.g. PM1 with pool We 6/97 and individual serum II 17). The inhibitory capacity of the modified allergens can be quantified by calculating the Prel values at 25% or 50% inhibition (1). The corresponding inhibitory values, and also the allergenic potency (Prel) measured at 25 or 50% inhibition, are shown for the serum pools and the individual sera in Tables 2 - 6.

Deletion mutants DM2 and DM2* show their loss of allergenicity by their Prel values, which are extremely low or can no longer be determined in a meaningful manner. While point mutations PM1 and PM3 exhibit a partial

loss of allergenicity, this loss is not adequate for practical use. Deletion mutants DM1 and DM3 exhibit a marked reduction in allergenicity. The reduction in IgE reactivity is superior to, or comparable with, that of the previously known chemically modified allergens and thereby makes these mutants particularly suitable candidates for immunotherapy.

Literature

Anderson MC and Baer H: Methodology for RAST inhibition. Food and Drug Administration, Bethesda, Maryland, U.S.A. (1986).

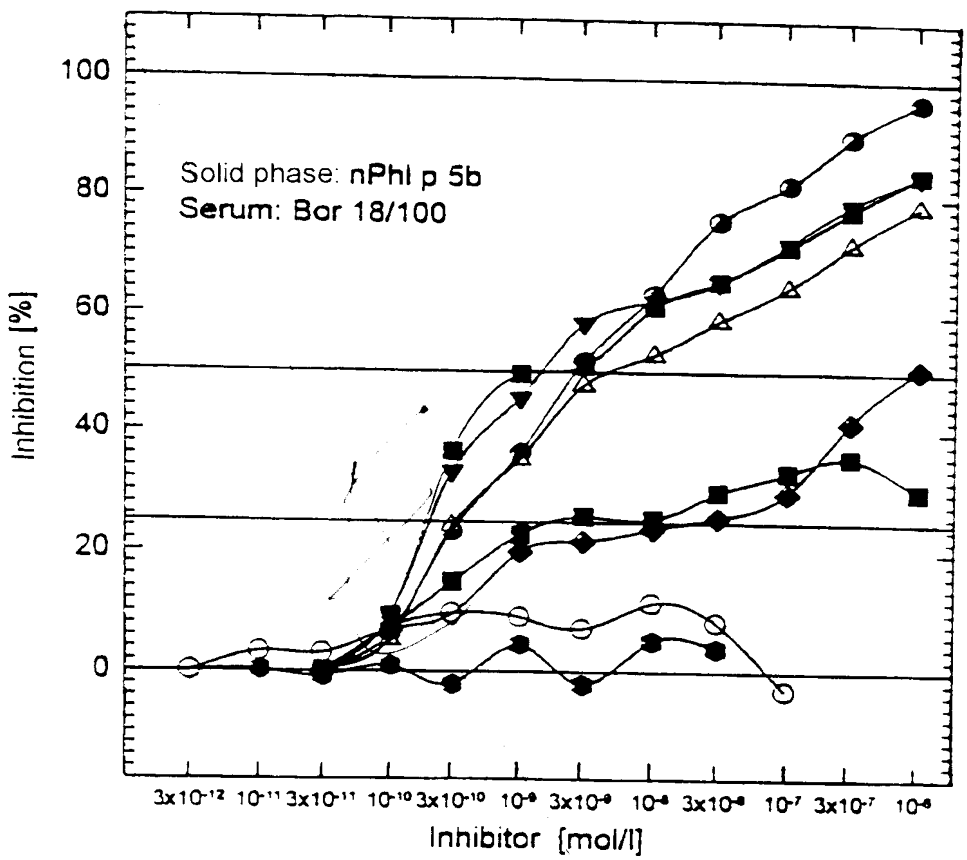


Figure 1 EAST inhibition curves of the Phl p 5b mutants using the allergic patient serum pool Bor 18/100

Inhibitors:

nPhl p 5b | PM 1 | DM 1 | DM 2
rPhl p 5b | PM 3 | DM 3 | DM 2*

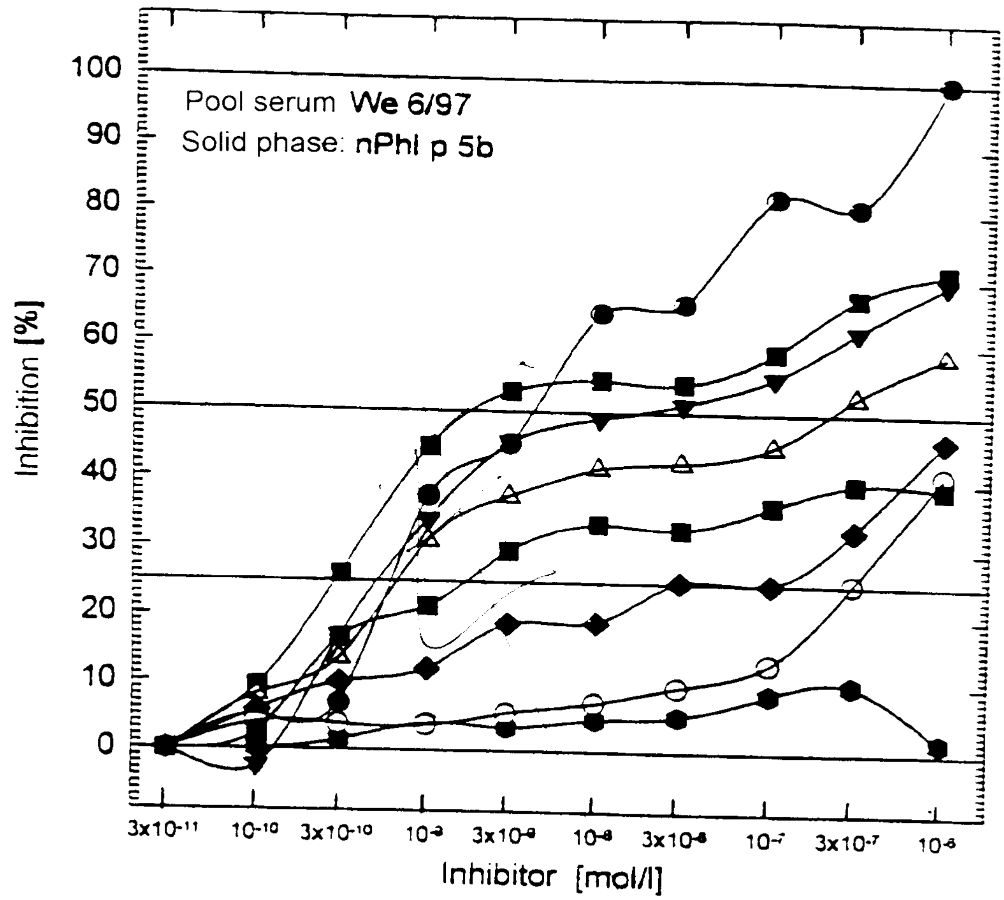
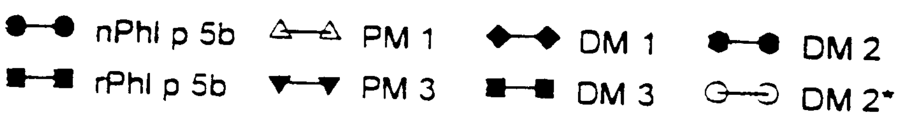


Figure 2 EAST inhibition curves of the Phl p 5b mutants using the allergic patient serum pool We 6/97

Inhibitors:



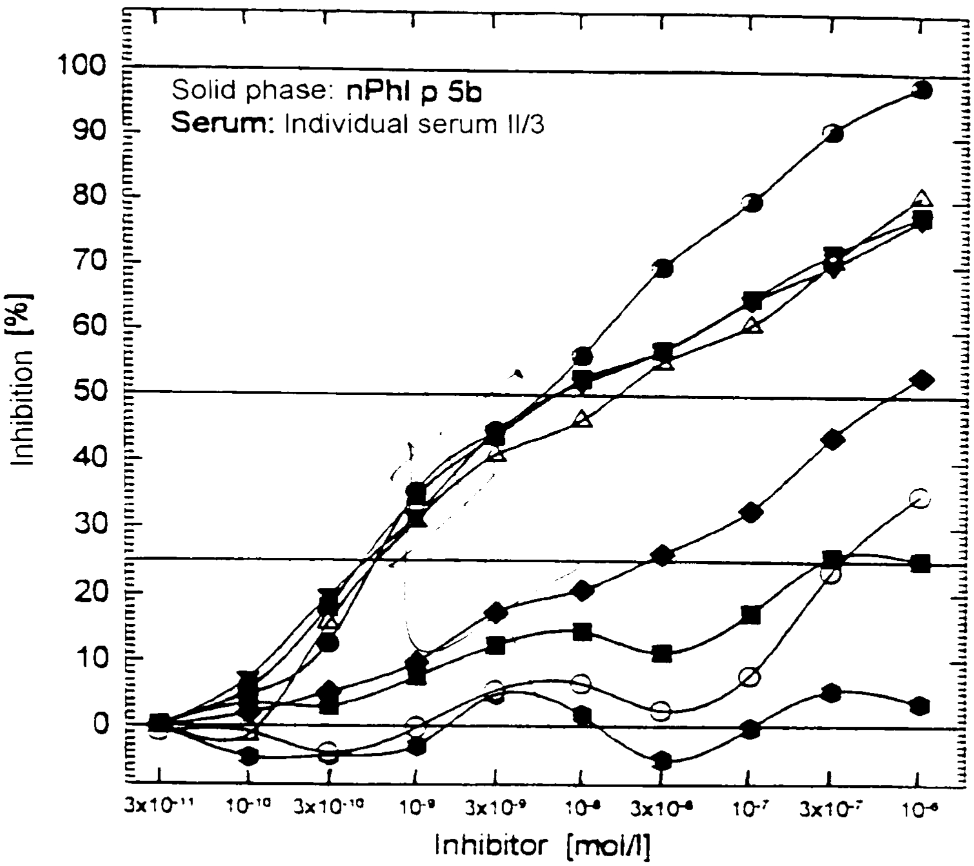


Figure 3 EAST inhibition curves of the Phl p 5b mutants using the individual allergic patient serum II/3

Inhibitors:

●—● nPhl p 5b　△—△ PM 1　◆—◆ DM 1　⬢—⬢ DM 2

■—■ rPhl p 5b　▼—▼ PM 3　■—■ DM 3　○—○ DM 2*

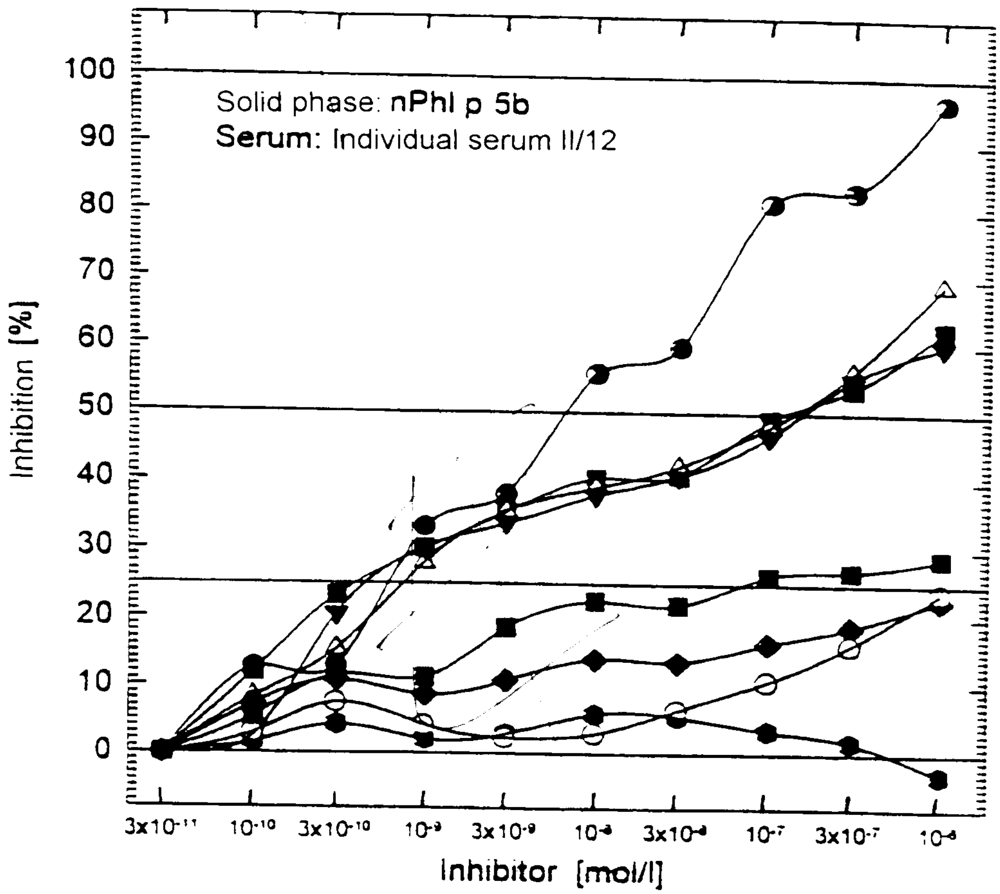
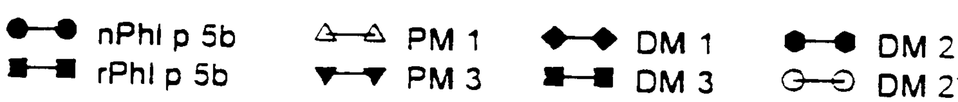


Figure 4 EAST inhibition curves of the Phl p 5b mutants using the individual allergic patient serum II/12

Inhibitors:



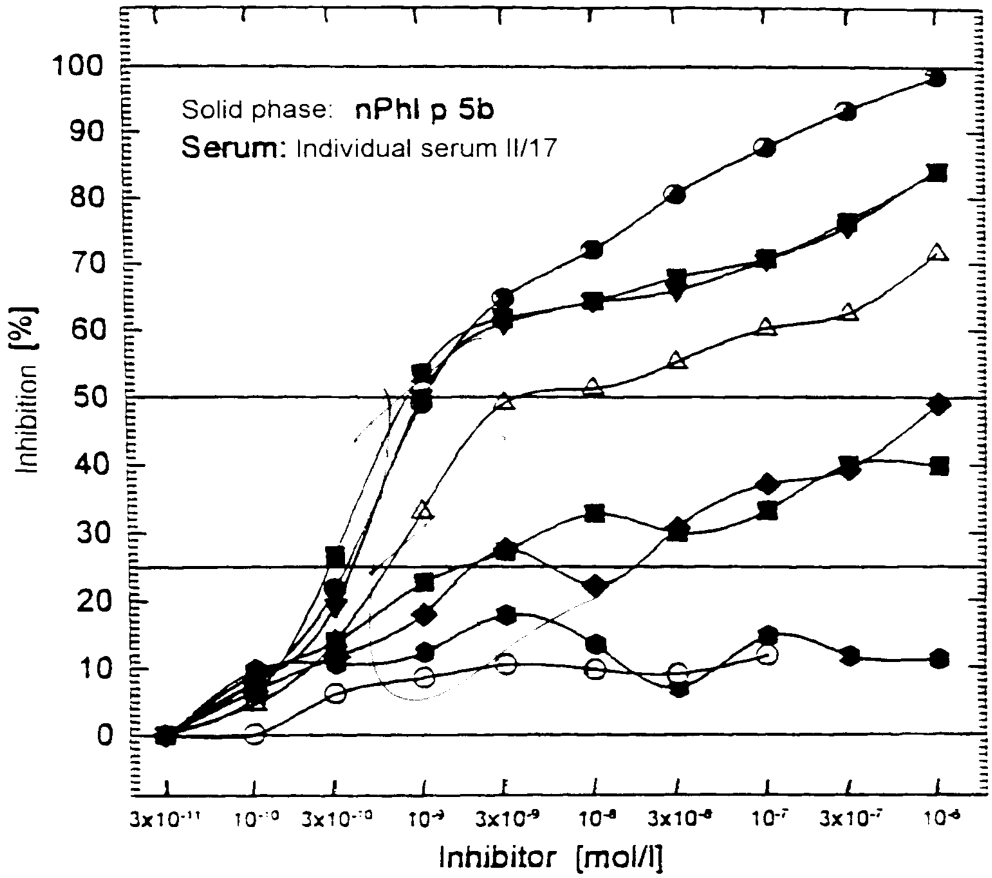


Figure 5 EAST inhibition curves of the Phl p 5b mutants using the individual allergic patient serum II/17

Inhibitors:

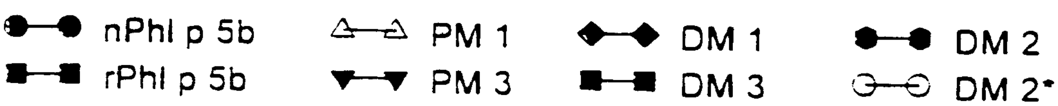


Table 2:
Allergenic potency ($P_{rel.}$) of the recombinant Phl p 5b mutants as compared with that of recombinant and native Phl p 5b using the allergic patient serum pool Bor 18/100

Inhibitor	Inhibition value[1] [mol/l]		Allergenic potency (P_{rel})[2]	
	25%	50%	25%	50%
n Phl p 5b	3.3×10^{-10}	4.2×10^{-9}	1.000	1.000
r Phl p 5b	2.0×10^{-10}	5.0×10^{-9}	1.709	0.8410
PM1	4.5×10^{-10}	1.2×10^{-8}	0.739	0.3490
PM3	2.0×10^{-10}	4.8×10^{-9}	1.641	0.8640
DM1	8.6×10^{-9}	2.8×10^{-8}	0.039	0.0015
DM2	8.3×10^{13}	2.3×10^{38}	4.0×10^{-23}	1.8×10^{-45}
DM3	1.2×10^{-8}	4.1×10^{-5}	0.028	0.0001
DM2*	5.0×10^{23}	2.3×10^{66}	6.7×10^{-34}	2.0×10^{-75}

[1] Inhibition values: Concentrations of the inhibitors at 25% and 50% inhibition, respectively

[2] Allergenic potency: Relative to native Phlp5b at 25% and 50% inhibition, respectively

Table 3:

Allergenic potency ($P_{rel.}$) of the recombinant Phl p 5b mutants as compared with that of recombinant and native Phl p 5b using the allergic patient serum pool We 6/97

Inhibitor	Inhibition value[1] [mol/l]		Allergenic potency (P_{rel})[2]	
	25%	50%	25%	50%
n Phl p 5b	5.1×10^{-10}	6.1×10^{-9}	1.000	1.000
r Phl p 5b	3.0×10^{-10}	1.4×10^{-8}	1.697	0.4400
PM1	1.2×10^{-9}	1.2×10^{-7}	0.415	0.0510
PM3	8.3×10^{-10}	3.0×10^{-8}	0.611	0.2030
DM1	2.3×10^{-8}	1.7×10^{-5}	0.022	0.0004
DM2	1.9×10^{8}	2.7×10^{21}	2.6×10^{-15}	2.3×10^{-30}
DM3	5.1×10^{-9}	2.9×10^{-8}	0.099	0.0020
DM2*	4.6×10^{-7}	1.5×10^{-3}	0.001	4.0×10^{-8}

[1] Inhibition values: Concentrations of the inhibitors at 25% and 50% inhibition, respectively

[2] Allergenic potency: Relative to native Phlp5b at 25% and 50% inhibition, respectively

Table 4:
Allergenic potency ($P_{rel.}$) of the recombinant Phl p 5b mutants as compared with that of recombinant and native Phl p 5b using the individual allergic patient serum II/3

Inhibitor	Inhibition value[1] [mol/l]		Allergenic potency $(P_{rel})^2$	
	25%	50%	25%	50%
n Phl p 5b	5.1×10^{-10}	5.9×10^{-9}	1.000	1.000
r Phl p 5b	$5.6 \cdot 10^{-10}$	$1.4 \cdot 10^{-8}$	0.9030	0.4190
PM1	8.6×10^{-10}	1.9×10^{-8}	0.5950	0.3140
PM3	5.5×10^{-10}	1.5×10^{-8}	0.9220	0.3990
DM1	1.2×10^{-8}	$1.7 \cdot 10^{-8}$	0.0420	0.0035
DM2	$6.6 \cdot 10^{10}$	$5.2 \cdot 10^{27}$	7.7×10^{-20}	1.1×10^{-38}
DM3	1.1×10^{-6}	0.032	0.0004	1.8×10^{-7}
DM2*	2.1×10^{-6}	0.010	0.0002	5.9×10^{-7}

[1] Inhibition values: Concentrations of the inhibitors at 25% and 50% inhibition, respectively

[2] Allergenic potency: Relative to native Phlp5b at 25% and 50% inhibition, respectively

Table 5:
Allergenic potency ($P_{rel.}$) of the recombinant Phl p 5b mutants as compared with that of recombinant and native Phl p 5b using the individual allergic patient serum II/12

Inhibitor	Inhibition value[1] [mol/l]		Allergenic potency (P_{rel})[2]	
	25%	50%	25%	50%
n Phl p 5b	$5.2 \cdot 10^{-10}$	$6.8 \cdot 10^{-9}$	1.000	1.000
r Phl p 5b	$8.7 \cdot 10^{-10}$	$7.3 \cdot 10^{-8}$	0.597	0.093
PM1	$1.3 \cdot 10^{-9}$	$8.3 \cdot 10^{-8}$	0.391	0.082
PM3	$1.3 \cdot 10^{-9}$	$9.1 \cdot 10^{-8}$	0.389	0.075
DM1	$1.5 \cdot 10^{-5}$	68.0	$3.4 \cdot 10^{-5}$	$1.0 \cdot 10^{-10}$
DM2	$3.8 \cdot 10^{10}$	$4.4 \cdot 10^{30}$	$1.4 \cdot 10^{-19}$	$1.6 \cdot 10^{-39}$
DM3	$4.5 \cdot 10^{-8}$	0.0001	0.012	$5.7 \cdot 10^{-5}$
DM2*	196.0	$7.4 \cdot 10^{14}$	$2.6 \cdot 10^{-12}$	$9.2 \cdot 10^{-25}$

[1] Inhibition values: Concentrations of the inhibitors at 25% and 50% inhibition, respectively

[2] Allergenic potency: Relative to native Phlp5b at 25% and 50% inhibition, respectively

Table 6:
Allergenic potency ($P_{rel.}$) of the recombinant Phl p 5b mutants as compared with that of recombinant and native Phl p 5b using the individual allergic patient serum II/17

Inhibitor	Inhibition value[1] [mol/l]		Allergenic potency (P_{rel})[2]	
	25%	50%	25%	50%
n Phl p 5b	2.2×10^{-10}	2.6×10^{-9}	1.000	1.000
r Phl p 5b	2.1×10^{-10}	4.7×10^{-9}	1.045	0.5450
PM1	6.4×10^{-10}	2.2×10^{-8}	0.336	0.1190
PM3	2.5×10^{-10}	5.5×10^{-9}	0.855	0.4680
DM1	6.5×10^{-9}	2.0×10^{-8}	0.033	0.0010
DM2	73.9	6.4×10^{19}	2.9×10^{-12}	4.1×10^{-29}
DM3	5.6×10^{-9}	5.0×10^{-8}	0.038	0.0005
DM2*	0.0004	11675.0	5.3×10^{-7}	2.2×10^{-13}

[1] Inhibition values: Concentrations of the inhibitors at 25% and 50% inhibition, respectively

[2] Allergenic potency: Relative to native Phlp5b at 25% and 50% inhibition, respectively

Example 5

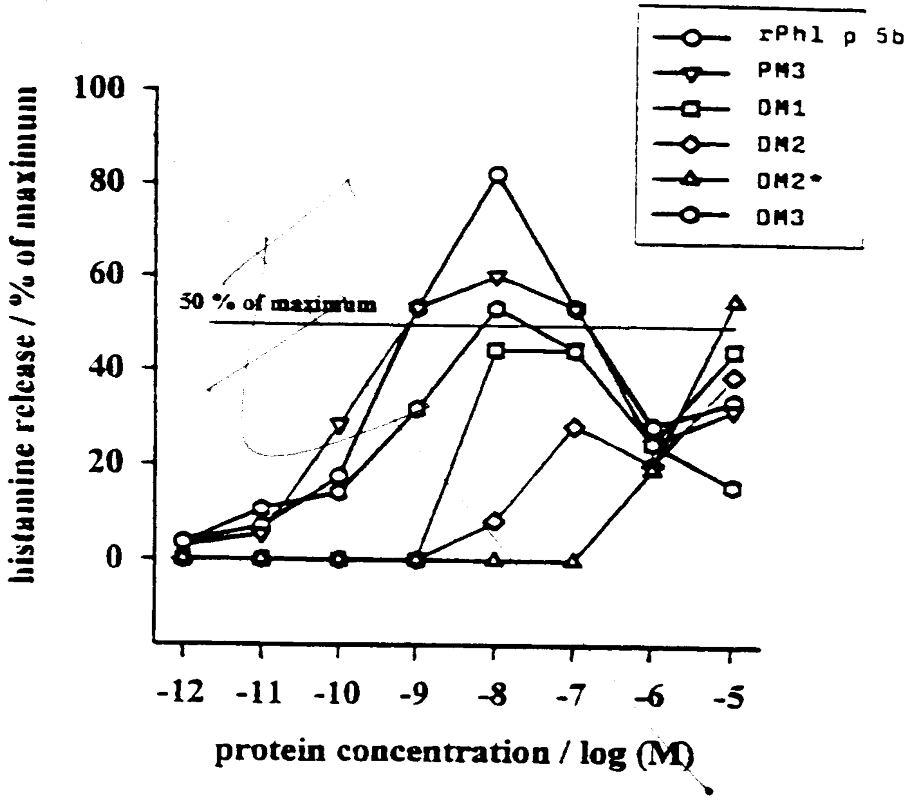
Reduced histamine release from basophils due to the rPhl p 5b mutants

The ability of the point mutant PM3 which was prepared, and of deletion mutants DM1, DM2, DM2* and DM3, to release histamine from basophils was tested and compared with that of the wild type rPhl p 5b.

Before the histamine release test was carried out, the basophilic leucocytes from the EDTA blood of an allergic patient (PS-W) were first of all enriched by means of dextran sedimentation and then adjusted to a final concentration of 100,000 basophils/ml. In order to release histamine from the basophils, 200 μl of the cell suspension were in each case incubated, at 37°C for 40 min, with 50 μl of antigen solution. For this, the rPhl p 5b and the mutants were employed in varying concentrations (of 10^{-5} - 10^{-12} M). The histamine which was released was determined in the respective supernatants using the Pharmacia methylhistamine RIA in accordance with the manufacturer's instructions.

In the histamine release test, all the recombinant proteins investigated described the typical bell-shaped curve as their concentrations increased (Fig. 6). The point mutant did not show any significant differences as compared with the wild type rPhl p 5b in its ability to release histamine. The concentrations of the deletion mutants DM3, DM1 and DM2 which were required to bring about a 30% histamine release were 3-fold, 20-fold and 500-fold higher, respectively. The deletion mutants therefore unambigously exhibit a decreased ability to release histamine from basophils.

Fig. 6 Release of histamine from human basophils after reaction with the allergens and allergen mutants



Example 6

Demonstration of the reactivity of recombinant Phl p 5b mutants with T cell clones from patients who are allergic to grass pollen

The reactivity of the recombinant Phl p 5b mutants was tested on established T cell clones (TCCs) of known specificity. The TCCs derive from patients who are allergic to grass pollen (see Ex. 1) and are directed against the T cell-reactive regions A (Fig. 7), B (Fig. 8) and C (Fig. 9). The T cell reactivity was measured by stimulating the clones to proliferate. The results clearly show that the TCCs react specifically with the Phl p 5b mutants when the relevant epitope is unaltered and, as expected, do not exhibit any reaction

when the epitope is missing or is altered by a point mutation.

Fig. 7: Proliferative reaction of Phl p 5b-reactive T cell clones (TCCs) from allergic patients with rPhl p 5b mutants

Specificity: Immunodominant T cell-reactive region A

Stimulator[1)]	Epitope present	Incorporation of $[^3H]$-thymidine into TCC[2)] JR 15	JR 13	CB 14	CB 2
Medium	-	-	-	-	-
n Phl p5	+	+++	+++	+++	+++
r Phl p5a	(±)	-	-	+	+
r Phl p5b	+	+++	+++	+++	+++
PM1	+	++	+++	+++	+++
PM3	+	+++	+++	+++	+++
DM1	+	+++	+++	+++	+++
DM2	+	+++	+++	nt [3)]	nt
DM2*	-	-	-	-	-
DM3	+	+++	+++	nt	nt
PL (12mer)	+	+++	+++	+++	+++

[1] Final concentration 0.3 μM

[2] Stimulation index SI: < 1 (-), 1-2 (+), 2-5 (+), 5-10 (++), > 10 (+++)

[3] n.t.: not tested

INS E4

Fig. 8: Proliferative reaction of Phl p 5b-reactive T cell clones (TCCs) from allergic patients with rPhl p 5b mutants

Specificity: Immunodominant T cell-reactive region B

Stimulator[1)]	Epitope present	Incorporation of $[^3H]$-thymidine into TCC[2)]	
		UZH2	DW8
Medium	-	-	-
n Phl p5	+	+++	+++
r Phl p5a	(±)	±	+++
r Phl p5b	+	+++	+
PM1	+	+++	+
PM3	+	+++	±
DM1	+	+++	+
DM2	-	-	-
DM2*	-	-	-
DM3	+	+++	+
PL (12mer)	+	+++	+++

[1] Final concentration 0.3 µM

[2] Stimulation index SI: < 1 (-), 1-2 (+), 2-5 (+), 5-10 (++), > 10 (+++)

[3] n.t.: not tested

INS E5

Fig. 9: Proliferative reaction of Phl p 5b-reactive T cell clones (TCCs) from allergic patients with rPhl p 5b mutants

Specificity: Immunodominant T cell-reactive region C

Stimulator[1]	Epitope present unchanged	Incorporation of $[^3H]$-thymidine[2]			
		1^{st} exp.	2^{nd} exp.	3^{rd} exp.	
Medium	-	1	1	1	-
n Phl p5	+	11.2	8.2	4.5	++
r Phl p5a	-	nt	<1	<1	-
r Phl p5b	+	11.0	7.0	5.5	++
PM1	-	<1	<1	1.1	-
PM3	+	7.4	5.9	4.5	++
DM1	+	8.6	6.2	4.4	++
DM2	+	14.4	9.1	7.1	+++
DM2*	+	12.8	12.1	11.7	+++
DM3	+	9.8	6.9	4.4	++
PL (12mer)	+	20.9	16.7	nt[3]	+++

[1] Final concentration 0.3 µM

[2] Stimulation index SI: < 1 (-), 1-2 (+), 2-5 (+), 5-10 (++), > 10 (+++)

[3] n.t.: not tested

Example 7

Testing the reactivity of recombinant Phl p 5b mutants with T cell lines from patients who are allergic to grass pollen

The oligoclonal T cell lines (TCLs) from 8 patients who were allergic to grass pollen (see Ex. 1) were established by repeated activation with natural Phl p 5b (a + b) or recombinant rPhl p 5b or 5a + 5b.

The proliferative reaction of these TCLs was tested using the rPhl p 5b mutants (Fig. 10). This shows that while all the mutants activate the TCLs, there are quantitative differences. The deletion mutant DM3 exhibits a strong specific stimulation with most of the TCLs.

INS E6

Fig. 10: Proliferative reaction of the Phl p 5b-reactive T cell lines (TCLs) from allergic patients with rPhl p 5b mutants

TCL	1 Wöl	2 Eic	3 Fre	4 Mer	6 Mah	5 17.4	7 19.2	8 20.1
	Incorporation of [^{3}H]-thymidine into TCLs[2]							
Primary stimulator	n Phl p 5	n Phl p 5	n Phl p 5	n Phl p 5	r Phl p 5a r Phl p 5b	r Phl p 5	r Phl p 5b	r Phl p 5b
Secondary activator[1]								
n Phl p 5	+++	+++	++	+++	+++	+	+++	+
r Phl p 5a	-	+	+	+	++	nt[3]	nt	nt
r Phl 5b	+	+	+	+	+++	+	+++	+++
PM1	+	±	+	±	++	+	+++	+++
PM3	±	±	+	+	±	+	+++	+
DM1	±	+	+	+	++	+	+++	+++
DM2	±	+	+++	+++	++	+	++	+++
DM2*	±	+	+	++	++	+	+	±
DM3	±	+	++++	++	+++	++	+++	+++

[1] Final concentration 0.3 μM

[2] Stimulation index SI: < 1 (-), 1-2 (+), 2-5 (+), 5-10 (++), > 10 (+++)

[3] n.t.: not tested

Recapitulative assessment of the results described in Examples 1 - 7

The mapping of the epitopes of the main allergen Phl p 5b which are recognized by T helper cells from patients who are allergic to grass pollen has demonstrated that the T cell epitopes of the individual T cell clones (TCLs) are distributed over the entire sequence of the Phl p 5b. However, **3 immunodominant T cell-reactive regions** which are recognized by 85% of the TCCs can be defined without difficulty (Example 1). It was possible to produce recombinant Phl p 5b mutants by means of point mutations (Example 2) and by means of deletion mutations (Example 3). The IgE reactivity of the point mutants (PM1 and PM3), as measured in the EAST inhibition test (Example 4), does not differ significantly from that of the wild-type Phl p 5b. While the IgE reactivity of the deletion mutants DM1 and DM3 is greatly reduced, it is still detectable. By contrast, the IgE binding of mutants DM2 and DM2* is very greatly reduced. This gradual decrease in the allergenicity of the rPhl p 5b mutants is also confirmed by the histamine release test using spec. IgE-loaded basophils from the blood of allergic patients (Example 5). The testing of the rPhl p 5b mutants with epitope-mapped T cell clones confirms that the point mutations and deletion mutations react with, or fail to stimulate, the TCCs in the expected manner (Example 6). Using oligoclonal T cell lines which were established from the blood of patients who are allergic to grass pollen by means of stimulation with Phl p 5, it was possible to demonstrate that the mutants are able to stimulate oligoclonal TCLs of this nature (Example 7). Taking the results of the reduction in allergenicity and the retention of the T cell stimulation together, the mutants, particularly the deletion mutants, constitute recombinant allergen variants which are potentially suitable for specific immunotherapy.

The following examples relate to pharmaceutical preparations:

Example A: Injection vials

A solution of 100 g of an active compound or of an active compound mixture based on the modified recombinant allergens and 5 g of disodium hydrogen phosphate in 3 l of doubly distilled water is adjusted to pH 6.5 with 2N hydrochloric acid, sterilized by filtration, aliquoted into injection vials and lyophilized under sterile conditions; the vials are then sealed in a sterile manner. Each injection vial comprises 5 mg of active compound.

Example B: Suppositories

A mixture of 20 g of an active compound in the form of the modified recombinant allergens together with 100 g of soya bean lecithin and 1400 g of cocoa butter is melted, poured into moulds and allowed to cool. Each suppository comprises 20 mg of active compound.

Example C: Solution

A solution of 1 g of an active compound in the form of the modified recombinant allergens, 9.38 g of $NaH_2PO_4 \cdot 2 H_2O$, 28.48 g of $Na_2HPO_4 \cdot 12 H_2O$ and 0.1 g of benzalkonium chloride is prepared in 940 ml of doubly distilled water. The solution is adjusted to pH 6.8, made up to 1 l and sterilized by irradiation. This solution can be used in the form of eye drops.

Example D: Ointment

500 mg of an active compound in the form of the modified recombinant allergens are mixed with 99.5 g of yellow soft paraffin under aseptic conditions.

Example E: Tablets

A mixture of 1 kg of active compound in the form of the modified recombinant allergens, 4 kg of lactose, 1.2 kg of potato starch, 0.2 kg of talc and 0.1 kg of magnesium stearate is compressed into tablets in the customary manner such that each tablet comprises 10 mg of active compound.

Example F: Coated tablets

Tablets are compressed in analogy with Example E and are then coated, in a customary manner, with a coating consisting of sucrose, potato starch, talc, gum tragacanth and dye.

Example G: Capsules

2 kg of active compound in the form of the modified recombinant allergens are aliquoted, in a customary manner, into hard gelatin capsules such that each capsule comprises 20 mg of the active compound.

Example H: Ampoules

A solution of 1 kg of active compound in the form of the modified recombinant allergens in 60 l of doubly distilled water is sterilized by filtration, aliquoted into ampoules and lyophilized under sterile conditions; the ampoules are then sealed in a sterile manner. Each ampoule comprises 10 mg of active compound.

Example I: Inhalation spray

14 g of active compound in the form of the modified recombinant allergens are dissolved in 10 l of an isotonic solution of NaCl and the solution is aliquoted into commercially available spraying vessels which are fitted with a pump mechanism. The solution can be

sprayed into the mouth or the nose. One spraying stroke (approximately 0.1 ml) corresponds to a dose of approximately 0.14 mg.